of the E-F-hand family of Calcium-sensing proteins. As shown in numerous studies, in vitro protein refolding can differ substantially from biosynthetic protein folding which already takes place co-translationally (1). One challenge is to characterize the adopted conformations of nascent chains before being released from the ribosome (2). CaM RNCs of full-length, half-length and of a structure consisting of the first E-F-hand only were synthesized in vitro. All constructs contained a tetra-cysteine motif site-specifically incorporated in the first N-terminal helix which is known to react with FlAsH, a biarsenic fluorescein derivative (3). As the dye is rotationally locked to this helix, the TRA decays should directly report on the rotational mobility of the investigated polypeptide chain. To investigate the scope of this procedure we analyzed TRA data of the different protein constructs free in solution as well as data of CaM with/without Calcium added. This enabled us to determine rotational correlation times, to choose suitable rotational diffusion models which fit the experimental data and thereby yield information about the conformational state and flexibility of the respective constructs. The feasibility of our approach to characterize structural properties and folding states of ribosome tethered, and released, polypeptide chains is finally discussed.

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Refined Folding Mechanism of a Helix-turn-helix Motif
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Helix-turn-helix motifs are valuable models for elucidating the protein folding mechanism. The *de novo* designed helix-turn-helix motif αtα has been studied thoroughly in our group by infrared spectroscopy and circular dichroism, applying site-specific isotopic labels and mutations. This preliminary investigation of αtα has provided detailed site-specific information on its folding mechanism, revealing increased stability in the middle of the helices and unfolding starting from the loose turn and the ends of the helices. The hydrophobic core contributes significantly to the stability of this little protein. To get further information on the folding mechanism of αtα, provided by additional independent probes, Förster resonance energy transfer (FRET) and differential scanning calorimetry (DSC) were employed to monitor thermal unfolding. FRET allows for measuring distances within a protein during its denaturation. In αtα, the intrinsic Trp residue in position 2 at the N-terminus and EDANS attached to the C-terminus served as FRET donor and acceptor, respectively, so end-to-end distances could be obtained. Thermodynamic parameters of a protein can be directly measured using DSC. Therefore, these directly obtained data were used to verify preliminary parameters from spectroscopic measurements. Overall, the results from FRET and DSC measurements were compared to the preliminary data on αtα to refine the proposed picture of its folding mechanism.

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Evidence for the Formation of Dry and Wet Molten Globules During Unfolding Process of a Small Protein
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The two-state model which is generally used to describe the 'first-order like' transitions of folding and unfolding of proteins is useful in evaluating thermodynamic parameters. However the implication of two-state model, viz. only native and unfolded forms exist is questionable when the complexity of the energy landscape is recognized.
Multiple steps observed during unfolding of a protein can provide information on the forces that maintain the folded structure. Solvation of the protein core determines stability, but it is not clear when such solvation occurs during unfolding. In this study, far-UV CD measurements suggest a simplistic two-state view of the unfolding of barstar, but the use of multiple probes brings out the complexity of the unfolding reaction. Near-UV CD measurements show that unfolding commences with the loosening of tertiary interactions resulting in a native-like intermediate, N*. FRET measurements show that N* then expands non-uniformly to form another intermediate, I_E. Spectral measurements of the single core tryptophan indicate that both N* and I_E retain native-like solvent accessibility of the core, suggesting that they are dry molten globules. Fluorescence quenching measurements suggest that the core then becomes solvated forming a wet molten globule, I_L, which precedes the unfolded form. Anisotropy decay measurements show that tight packing around tryptophan is lost when I_L forms. Strikingly, the slowest step is unfolding of the wet molten globule, and involves a solvated transition state.

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Volume of Hsp90 Ligand Binding and the Unfolding Phase Diagram as a Function of Pressure and Temperature
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The phenomenon of inhibitor binding to protein plays a major role in drug discovery. A complete thermodynamic picture of protein unfolding and ligand binding processes can only be obtained if both pressure- and temperature-induced protein denaturation techniques are utilized. The mechanism by which high pressure destabilizes proteins is poorly understood, but there is an agreement that pressure is an equally important fundamental thermodynamic variable as temperature. Temperature-induced protein unfolding provides information about enthalpy and entropy while pressure-induced protein unfolding - about volumetric properties of the system.
In this work, we present thermodynamic characterization of heat shock protein 90 (Hsp90) ligand binding and the unfolding phase diagram as a function of pressure and temperature. Hsp90 is an ATPase and a molecular chaperone responsible for the correct folding of client proteins. Hsp90 is essential for tumor progression because it maintains client proteins in their active forms. Inhibition of Hsp90 leads to the simultaneous degradation of these oncogenic proteins. For this reason Hsp90 has become an anticancer drug target. Hsp90 stability, ligand binding volume, enthalpy, entropy and affinity were measured by combining the Pressure shift assay, Thermal shift assay, and Isothermal titration calorimetry. The study has shown that the ligand increased both the melting pressure and melting temperature, and protein-ligand binding affinity may be correlated with binding volumes.

3401-Pos Board B129
Origin of Enthalpic Depletion Forces
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Solutes preferentially excluded from macromolecules can drive depletion attractions in important biological and colloidal association processes. The established Asakura-Oosawa theory relates depletion forces to the reduction in excluded volume and the ensuing entropy gain upon macromolecular compaction. In line with this theory, cosolute-induced protein stabilization or favored protein association are often described in terms of entropically driven ''crowding'', a specific manifestation of depletion interactions. In agreement, our recent experiments of peptide folding and supramolecular binding suggest that depletion forces are predominantly entropic for some cosolutes, such as polyethylene glycol polymers. Surprisingly, however, for other solutes such as polyol osmolytes, the main thermodynamic contribution is enthalpic, while the entropic change due to cosolutes can even be unfavorable. To further elucidate the molecular basis of this enthalpic depletion interaction, we have been using both simulations and analytic theory. Monte-Carlo simulations follow the association of two rod ''macromolecules'' in binary Lennard-Jones solutions. By dissecting the free energy change upon approach of the two macromolecules into the respective enthalpic and entropic components, we find that different cosolutes show distinct contributions to their macromolecular stabilization effect, implying different thermodynamic driving mechanisms. When considering intermolecular interactions beyond hard-cores, not all cosolutes conform to the established model, and even for these simple, nonassociative liquids, depletion forces can be completely enthalpic in nature. We discuss and analyze this newly resolved mechanism for depletion forces that originates from intermolecular interactions and solvent restructuring. Finally, a mean-field theoretical model based on the Flory-Huggins solution theory complements the simulation analysis.

3402-Pos Board B130
Statistical Mechanical Models for Analyzing the Site-Specific Folding of helix-turn-helix Motifs
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Isotopically-edited IR spectroscopy can provide detailed site-specific information about the protein folding mechanism. With our equilibrium unfolding studies of two simple helix-turn-helix (hth) proteins using circular dichroism and ^{13}C isotopically labeled infrared spectroscopy, we incorporated this